Transgenic mice having a human major histocompatibility complex (MHC) phenotype, experimental uses and applications

CROSS-REFERENCE TO RELATED APPLICATION

[001]   This application is based on and claims the benefit of U.S. Provisional Application No. 60/490,945, filed July 30, 2003 (Attorney Docket No. 03495.6093), the entire disclosure of which is relied upon and incorporated by reference herein for all purposes.

BACKGROUND OF THE INVENTION

[002]   Many vaccines are currently being developed for human cancer immunotherapy and for treatment of infectious diseases, such as malaria, AIDS, hepatitis C virus, and SARS. Given the rapidity with which new emerging pathogens can appear, it is important to improve animal models that could be used to evaluate vaccination strategies and the protective capacity of different epitopes quickly and reliably. Furthermore, *in vivo* studies are already required to assess crucial variables of vaccine behavior that are not easily evaluated or impossible to measure *in vitro*, such as vaccine immunogenicity, vaccine formulation, route of administration, tissue distribution, and involvement of primary and secondary lymphoid organs. Because of their simplicity and flexibility, small animals, such as mice represent an attractive alternative to more cumbersome and expensive model systems, such as nonhuman primates, at least for initial vaccine development studies.

[003] The moderate efficacy observed in several clinical trials of vaccines, which were found to be protective in wild-type animal studies (McMichael, A.J. & Hanke, T. Nat Med 9, 874-880 (2003)), may be partly explained by the different influence that human and animal MHC have on the outcome of the immune response, since animal MHC and human HLA molecules do not present the same optimal epitopes (Rotzschke, O. et al. Nature 348, 252-254 (1990)). Thus, despite some limitations, transgenic mice expressing human HLA should represent a useful improvement over wild-type mice as a preclinical model for testing vaccine candidates, evaluating the potential risk that the vaccines could induce autoimmune disorders, and devising better therapeutic strategies based on the human restriction element.

Cytotoxic T Cells

[004] Cytotoxic T cells (CTL) play a crucial role in the eradication of infectious diseases and in some cases, cancer (P. Aichele, H. Hengartner, R.M. Zinkernagel and M. Schulz, J Exp Med 171 (1990), p. 1815; L. BenMohamed, H. Gras-Masse, A. Tartar, P. Daubersies, K Brahimi, M. Bossus, A. Thomas and P. Druhile, Eur J Immunol 27 (1997), p. 1242; D.J. Diamond, J. York, J. Sun, C.L. Wright and S.J. Forman, Blood 90 (1997), p. 1751). Recombinant protein vaccines do not reliably induce CTL responses (Habeshaw JA, Dalgleish AG, Bountiff L, Newell AL, Wilks, D, Walker LC, Manca F. 1990 Nov; 11(11): 418-25; Miller SB, Tse H, Rosenspire AJ, King SR. Virology. 1992 Dec; 191 (2):9 73-7). The use of otherwise immunogenic vaccines consisting of attenuated pathogens in humans is hampered, in several important diseases, by overriding safety concerns. In the last few years, epitope-based approaches have been

proposed as a possible strategy to develop novel prophylactic and immunotherapeutic vaccines (Melief CJ, Offringa R, Toes RE, Kast WM. Curr Opin Immunol. 1996 Oct, 8(5):651-7; Chesnut RW, Design testing of peptide based cytotoxic T-cell mediated immunotherapeutic to treat infiction disease, cancer, in Ppowell, MF, Newman, MJ (eds.): Vaccine Design: The Subunit, Adjuvant Approach, Plenum Press, New-York 1995, 847). This approach offers several advantages, including selection of naturally processed epitopes, which forces the immune system to focus on highly conserved and immunodominant epitopes of a pathogen (R.G. van der Most, A. Sette, C. Oseroff, J. Alexander, K. Murali-Krishna, L.L. Lau, S, Southwood, J. Sidney, R.W. Chesnut, M. Matioubian and R. Ahmed, J Immunol 157 (1996), p. 5543) and induction of multiepitopic responses to prevent escape by mutation such observed in HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. It also allows the elimination of suppressive T cell determinants, which might preferably elicit a TH2 response, in conditions where a TH1 responses is desirable, or vice-versa (Pfeiffer C, Murray J, Madri J, Bottomly K. Immunol Rev. 1991 Oct;123:65-84; P Chaturvedi, Q Yu, S Southwood, A Sette, and B Singh Int Immunol 1996 8: 745-755). It finally provides the possibility to get rid of autoimmune T cell determinants in antigens, which might induce undesirable autoimmune diseases. Protective antiviral or anti-tumoral immunity using CTL epitope-peptides has been achieved in several experimental models (D.J. Diamond, J. York, J. Sun, C.L. Wright and S.J. Forman, Blood 90 1997, p. 1751; J.E.J. Blaney, E. Nobusawa, M.A. Brehm, R.H. Bonneau, L.M. Mylin, T.M. Fu, Y. Kawaoka and S.S. Tevethia, J Virol 72 (1998), p. 9567).

[005] CTL epitope definition based on the usage of human lymphocytes might be misleading due to environmental and genetic heterogeneity that lead to incomplete results, and due to technical difficulties in isolating CTL clones. HLA class I or class II transgenic mice described to date have proved to be a valuable tool to overcome these limitations as illustrated by the identification with such animal models of novel CTL and T helper epitopes (Hill AV. Annu Rev Immunol. 1998;16:593-617; Carmon L, El-Shami KM, Paz A., Pascolo S, Tzehoval E, Tirosb B, Koren R, Feldman M, Fridkin M, Lemonnier FA, Eisenbach L. Int J Cancer, 2000 Feb 1;85(3):391-7). These mice have also been used to demonstrate: i) good correlation between peptide HLA binding affinity and immunogenicity (Lustgarten J, Theobald M, Labadie C, LaFace D, Peterson P, Disis ML, Cheaver MA, Sherman LA. Hum Immunol. 1997 Feb;52(2):109-18; Bakker AB, van der Burg SH, Huijbens RJ, DRijfhout JW, Melief CJ, Adema GJ, Figdor CG. Int J Cancer. 1997 Jan 27;70(3):302-9), ii) significant overlap between the murine and human CTL system at the level of antigen processing (same epitopes generated), and iii) comparable mobilization against most antigens of the CTL repertoires in HLA transgenic mice and humans (Wentworth, P. A., A. Vifiello, J. Sidney, E. Keogh, P, W. Chesnut, H. Grey, A. Sette. 1996. Eur. J. Immunol. 26:97; Alexander, J., C. Oserof, J. Sidney, P. Wentworth, E. Keogh, G. Hermanson, F. V. Chisari R. T, Kubo, H. M, Grey, A, Sette, 1997. J.Immunol. 159:4753).

[006] To date, synthetic peptide-based CTL epitope vaccines have been developed as immunotherapeutics against a number of human diseases [18-20]. However, only moderate efficacy was observed in several clinical trials (21). This may be partly explained by the failure of these vaccines to induce sufficiently strong CTL

responses. Indeed, recent reports suggest the need for CD4+ T-cell help to obtain maximum CTL response (A.J. Zajac, K. Murali-Krishna, J.N. Blattman and R. Ahmed, Curr Opin Immunol 10 (1998), p. 444; Firat H, Garcia-Pons F, Tourdot S, Pascolo S, Scardino A, Garcia Z, Michel ML, Jack RW, Jung 0, Kosmatopoulos K, Mateo L, Suhrbier A, Lemonnier FA, Langlade-Demoyen P Eur J Immunol 29, 3112, 1999).

[007] CTL are critical components of protective immunity against viral infections, but the requirements for *in vivo* priming of CTL are not completely understood. It is now accepted that Th cells are usually essential for CTL priming with synthetic peptides. With respect to synthetic CTL epitopic peptides, several studies point to a mandatory need for Th lymphocyte stimulation to induce optimal CTL responses (C. Fayolle, E. Deriaud and C. Leclerc, J Immunol 147 (1991), p, 4069; C. Widmann, P. Romero, J.L. Maryanski, G. Corradin and D. Valmori, J Immunol Meth 155 (1992), p. 95; M. Shirai, C.D. Pendkton, J. Ahlers, T. Takeshita, M. Newman and J.A. Berzofsky, J Immunol 152 (1994), p. 549; J.P. Sauet, H. Gras-Masse, J.G. Guillet and E. Gomard, Int Immunol 8 (1996). p. 457). Several of these studies showed that activation of a CD8+ T cell requires simultaneous interaction of a CD4+ T helper cell and a CD8+ T cell with the same antigen-presenting cell presenting their cognate epitopes (Ridge JP, Di Rosa F, Matzinger P. Nature. 1998 Jun 4;3 93 (6684):474-8). The relevance of this three-cell interaction for priming of CTLs is confirmed by studies with viral epitopes, and animal models, since *in vivo* induction of CTLs was most efficient when CTL and Th epitopes were physically linked rather than administered as an unlinked mixture (Shirai M, Pendleton CD, Ahlers J, Takeshita T, Newman M, Berzohky JA. J Immunol. 1994 Jan 15;152(2): 549-56; Oseroff C, Sette A, Wentworth P, Celis E, Maewal A, Dahlberg C,

Fikes J, Kubo RT, Chesnut RW, Grey BX Alexander J. Vaccine. 1998 May;16(8): 823-

33). The capacity of CTL and Th antigenic peptides to efficiently induce CTL responses

has been demonstrated both in experimental models (C. Fayolle, E. Deriaud and C.

Leclerc, J Immunol 147 (1991),. p, 4069; C. Widmann, P. Romero, J.L. Maryanski, G.

Corradin and D. Valmori, J Immunol Meth 155 (1992), p. 95) and in humans (A. Vitiello,

G. Ishioka, H.M. Grey, R. Rose, P. Famess, R. LaFond, L. Yuan, F.V. Chisari, J. Furze

and R. Bartholomeuz, J Clin Invest 95 (1995), p. 341; B. Livingston, C. Crimi, H. Grey,

G. Ishioka, F.V. Chisari, J. Fikes, H.M. Grey, R. Chesnut and A. Sette, J Immunol 159

(1997), p. 1383). Moreover, a potent Th response plays an important role not only for

optimal induction of CTL responses, but also for maintenance of CTL memory (E.A.

Walter, P.D. Greenberg, M.J. Gilbert, R.J. Finch, K-S. Watanabe, E.D. Tbomas and

S.R. Riddell, N Engl J Med 333 (1995), p. 1038; Riddell SR, Greenberg PD, In Thomas

ED, Blume KG, Forman SJ (eds): Hematopoietic Cell Transplantation, 2nd edn. Malden,

MA: Blackwell Science Inc., 1999). Finally, it has long been documented that CD4+ T

"helper" cells are crucial in coordinating cellular and humoral immune responses against

exogenous antigens.

[008]   Recently, a transgenic (Tg) mouse that expresses both HLA-A*0201 class

I and HLA-DR1 class II molecules was established (BenMohamed L, Krishnan R,

Longmate J, Auge C, Low L, Primus J, Diamond DJ, Hum, Immunol. 2000

Aug;61(8):764-79). The authors reported that both HLA-A*0201 and HLA-DR1

transgenes are functional *in vivo*, that both MHC class I and class II molecules were

utilized as restriction elements, and that the product of the HLA-DR1 transgene

enhances the HLA-A*0201-restricted antigen-specific CTL responses (BenMohamed L,

Krishnan R, Longmate J, Auge C, Low L, Primus J, Diamond DJ, Hum, Immunol. 2000 Aug;61(8):764-79).

[009] It is noteworthy that these HLA-A*0201/DR1 Tg mice expressed their own MHC H-2 class I and class II molecules. Because HLA class I transgenic mice expressing endogenous mouse MHC class I genes preferentially and often exclusively develop H-2 restricted CTL response (C Barra, H Goumier, Z Garcia, PN Marche, E Jouvin-Marche, P Briand, P Fillipi, and FA Lemonnier J Immunol 1993 150: 3681-3689; Epstein H, Hardy F., May JS, Johnson MH, Holmes N. Eur J Immunol. 1989 Sep;19(9):1575-83; Le AX; EJ Bernhard, MJ Holterman, S Strub, P Parham, E Lacy, and VH Engelhard J Immunol 1989 142: 13 66-1371; Vitiello A, Marchesini D, Furze J, Sherman LA, Chesnut RW., J Exp Med. 1991 Apr 1;173(4):100715), and HLA class II transgenic mice expressing endogenous mouse MHC class II genes fail to induce reliable HLA class II restricted antigen-specific responses (Nishimura Y, Iwanaga T, Inamitsu T, Yanagawa Y, Yasunami M, Kimura A, Hirokawa K, Sasazuki T., J Immunol 1990 Jul 1;145(I):353-60), these HLA-A*0201/DR1 Tg mice are of limited utility to assess human-specific responses to antigen.

[010] However, in HLA class I transgenic H-2 class I knock-out mice, or HLA class II transgenic H-2 class II knock-out mice, only HLA-restricted CTL immune responses occur (Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau, B., J Exp Med. 1997 Jun 16;185(12).2043-51; Madsen L, Labrecque N, Engberg J, Dierich A, Svejgaard A, Benoist C, Mathis D, Fugger L. ,Proc Natl Acad Sci U S A- 1999 Aug 31;96(18):10338-43). In fact, *HLA-A2.1*-transgenic *H-2 class I*-knock-out (KO) mice exhibit the ability to mount enhanced HLA-A2.1-restricted responses as compared

to *HLA-A2.1*-transgenic mice that still express the endogenous murine H-2 class I molecules (Pascolo, S. et al. *J Exp Med* 185, 2043-2051 (1997); Ureta-Vidal, A., Firat, H., Peramau, B. & Lemonnier, F.A. *J Immunol* 163, 2555-2560 (1999); Firat, H. et al., *Int Immunol* 14, 925-934 (2002); Rohrlich, P.S. et al., *Int Immunol* 15, 765-772 (2003)). The inventors have made similar observations with *HLA-DR1*-transgenic mice, depending on whether or not they are deficient in H-2 class II molecules (A. Pajot, unpublished results). Furthermore, in the absence of competition from murine MHC molecules, the *HLA-A2.1*-transgenic *H-2 class I*-KO or *HLA-DR1*-transgenic *H-2 class II*-KO mice generate only HLA-restricted immune responses (Pascolo, S. et al. *J Exp Med* 185, 2043-2051 (1997)) (A. Pajot, unpublished results), facilitating the monitoring of HLA-restricted $CD8^+$ and $CD4^+$ T cell responses. However, protective immune responses against pathogens, which often require collaboration between T helper and cytotoxic $CD8^+$ T cells, cannot be studied in the single *HLA class I*- or *HLA class II*-transgenic mice, which do not allow the simultaneous assessment of HLA class I and II human responses in the same mouse.

[011] Accordingly, there exists a need in the art for a convenient animal model system to test the immunogenicity of human vaccine candidates comprising constructs containing human CTL epitopes and, in some cases, with the inclusion of high potency CD4+ Th (helper T lymphocyte) epitopes to sustain antiviral and antitumoral CD8+ T-cell activity (A.J. Zajac, K. Murali-Krishna, J.N. Blattman and R. Ahmed, Curr Opin Immunol 10 (1998), p. 444; Firat H, Garcia-Pons F, Tourdot S, Pascolo S, Scardino A, Garcia Z, Michel ML, Jack RW, Jung 0, Kosmatopoulos K, Mateo L, Suhrbier A, Lemonnier FA, Langlade-Demoyen P, Eur J Irmnunol 29,3112, 1999). There is also a

need for a system that allows the simultaneous assessment of the mutual coordination between a CTL response, a TH response (in particular s TH_1 or TH_2 response), and, optionally, a humoral response.

SUMMARY OF THE INVENTION

[012] The inventors have met this need and more by providing mice transgenic for both HLA-A2.1 and HLA-DR1 molecules, in a background that is deficient for both H-2 class I and class II molecules. Specifically, the invention provides mice comprising (1) mutated H-2 class I and class II molecules; and (2) expressing HLA class I transgenic molecules, or HLA class II transgenic molecules, or HLA class I transgenic molecules and HLA class II transgenic molecules. These mice provide a model useful in the development and optimization of vaccine constructs with maximum *in vivo* immunogenicity for human use. Specifically, such mice enable a complete analysis of the three components of the immune adaptive response (antibody, helper and cytolytic) in a single animal, as well as an evaluation of the protection specifically conferred by vaccination against an antigenic challenge.

[013] Mice of the invention, which comprise a knock-out for both H-2 class I and class II molecules, and express HLA class I transgenic molecules and HLA class II transgenic molecules represent a completely humanized experimental mouse that can be used to simultaneously detect the presence of antigen-specific antibodies, an antigen-specific HLA–DRI restricted T cell response, and an antigen-specific HLA-A2 restricted T cell response. These mice will be useful to study how mutual coordination operates between a CTL response, a TH response (in particular a TH_1 or TH_2

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response), and, optionally, a humoral response. These mice represent an optimized tool for basic and applied vaccinology studies.

[014] A first embodiment of the invention provides a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, and a functional HLA class I or class II transgene.

[015] A second embodiment of the invention provides a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA class I transgene, and a functional HLA class II transgene.

[016] In some embodiments, the HLA class I transgene is an HLA-A2 transgene and the HLA class II transgene is an HLA-DR1 transgene. In other embodiments, the HLA-A2 transgene comprises the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene comprises the HLA-DR1 sequence provided in the sequence listing.

[017] A further embodiment of the invention provides a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene. In an embodiment, the mouse has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m$^\circ$IAβ°. In some embodiments the HLA-A2 transgene comprises the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene comprises the HLA-DR1 sequence provided in the sequence listing.

[018] Another embodiment of the invention provides a method of simultaneously identifying the presence of one or more epitopes in a candidate antigen or group of antigens, where the one or more epitopes elicits a specific humoral response, a TH

HLA-DR1 restricted response, and/or a CTRL HLA-A2 restricted response. The method comprises administering the candidate antigen or group of candidate antigens to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m^{o}IAβ^o; assaying for a specific humoral response in the mouse to the antigen; assaying for a TH HLA-DR1 restricted response in the mouse to the antigen; and assaying for a CTRL HLA-A2 restricted response in the mouse to the antigen. Observation of a specific humoral response in the mouse to the antigen identifies an epitope that elicits a humoral response in the antigen. Observation of a TH HLA-DR1 restricted response in the mouse to the antigen identifies an epitope that elicits a TH HLA-DR1 restricted response in the antigen. Observation of a CTRL HLA-A2 restricted response in the mouse to the antigen identifies an epitope which elicits a CTRL HLA-A2 restricted response in the antigen.

[019] In some embodiments, the method includes assaying for a Th1-specific response in the mouse to the antigen and assaying for a Th2-specific response in the mouse to the antigen. In this case, observation of a Th1-specific response in the mouse to the antigen identifies an epitope that elicits a Th1-specific response in the mouse to the antigen, and observation of a Th2-specific response in the mouse to the antigen identifies an epitope that elicits a Th2-specific response in the mouse to the antigen.

[020] This invention also provides a method of identifying the presence of an HLA DR1-restricted T helper epitope in a candidate antigen or group of candidate

antigens, the method comprising administering the candidate antigen or group of candidate antigens to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$$\beta$2m^{o}IA$\beta^o$; and assaying for a TH HLA-DR1 restricted T helper epitope response in the mouse to the antigen. Observation of a TH HLA-DR1 restricted T helper epitope response in the mouse to the antigen identifies an epitope that elicits a TH HLA-DR1 restricted T helper epitope response in the antigen.

[021] In addition, this invention provides an isolated antigen comprising an HLA DR1-restricted T helper epitope identified by the method of the preceding paragraph. In some embodiments, the isolated antigen further includes an epitope that elicits a humoral response and/or an epitope that elicits a CTRL HLA-A2 restricted response. In some embodiments, the antigen comprising an HLA DR1-restricted T helper epitope comprises a polypeptide. In other embodiments, the antigen comprising an HLA DR1-restricted T helper epitope comprises a polynucleotide. In further embodiments, the antigen comprising an HLA DR1-restricted T helper epitope comprises DNA, RNA, or DNA and RNA.

[022] Further, this invention provides a method of identifying the presence of an HLA-A2-restricted T cytotoxic (CTL) epitope in a candidate antigen or group of candidate antigens, the method comprising administering the candidate antigen or group of candidate antigens to a transgenic mouse comprising a disrupted H2 class I

gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°; and assaying for an HLA-A2-restricted T cytotoxic (CTL) response in the mouse to the antigen or group of antigens. Observation of an HLA-A2-restricted T cytotoxic (CTL) response in the mouse to the antigen or group of antigens identifies an epitope that elicits a an HLA-A2-restricted T cytotoxic (CTL) response in the antigen or group of antigens.

[023] This invention provides an isolated antigen comprising an HLA-A2-restricted T cytotoxic (CTL) epitope identified by the method of the preceding paragraph. In some embodiments, the antigen further comprises an epitope that elicits a humoral response and/or an epitope that elicits a TH HLA-DR1 restricted T helper epitope response. In some embodiments, the antigen comprising an HLA-A2-restricted T cytotoxic (CTL) epitope comprises a polypeptide. In other embodiments, the antigen comprising an HLA-A2-restricted T cytotoxic (CTL) epitope comprises a polynucleotide. In further embodiments, the antigen comprising an HLA-A2-restricted T cytotoxic (CTL) epitope comprises, DNA, RNA, or DNA and RNA.

[024] This invention also provides a method of comparing the efficiency of the T-helper cell response induced by two or more vaccines. This method comprises administering a first candidate vaccine to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and

class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m°IAβ°, and measuring the T-helper cell response induced in the mouse by the first candidate vaccine; administering a second candidate vaccine to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m°IAβ°, and measuring the T-helper cell response induced in the mouse by the second candidate vaccine; administering each additional candidate vaccine to be compared to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m°IAβ°, and measuring the T-helper cell response induced in the mouse by the additional candidate vaccine, and determining the efficiency of each candidate vaccine to induce a T-helper cell response by comparing the T-helper cell responses to each of the vaccines to be compared with each other. In some embodiments the T-helper cell response is an HLA-DR1 restricted response.

[025] In addition, this invention provides a method of comparing the efficiency of T cytotoxic cell responses induced by two or more vaccines. The method includes

administering a first candidate vaccine to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°, and measuring the T cytotoxic cell response induced in the mouse by the first candidate vaccine; administering a second candidate vaccine to a mouse of a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°, and measuring the T cytotoxic cell response induced in the mouse by the second candidate vaccine; administering each additional candidate vaccine to be compared to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°, and measuring the T cytotoxic cell response induced in the mouse by the additional candidate vaccine; and determining the efficiency of each candidate vaccine to induce a T cytotoxic cell response by comparing the T cytotoxic cell responses to each of the vaccines to be compared with

each other. In some embodiments the T cytotoxic cell response is an HLA-A2 restricted response.

[026]   Further, this invention provides a method of simultaneously comparing the efficiency of T-helper cell response and T cytotoxic cell response induced by two or more vaccines. The method comprises administering a first candidate vaccine to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m$^\circ$IAβ°, and measuring the T-helper cell response and T cytotoxic cell response induced in the mouse by the first candidate vaccine; administering a second candidate vaccine to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta$2m$^\circ$IAβ°, and measuring the T-helper cell response and T cytotoxic cell response induced in the mouse by the second candidate vaccine; administering each additional candidate vaccine to be compared to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene; a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II

transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°, and measuring the T-helper cell response and T cytotoxic cell response induced in the mouse by each additional candidate vaccine; and determining the efficiency of each candidate vaccine to induce a T-helper cell response and T cytotoxic cell response by comparing the T-helper cell response and T cytotoxic cell response to each of the vaccines to be compared with each other. In some embodiments the T-helper cell response is an HLA-DR1 restricted response, and the T cytotoxic cell response is an HLA-A2 restricted response.

[027] This invention also provides a method of simultaneously determining the humoral response, the T-helper cell response, and the T cytotoxic cell response of a mouse following its immunization with an antigen or a vaccine comprising one or more antigens. The method comprises administering the antigen or the vaccine comprising one or more antigens to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°, and assaying for a specific humoral response in the mouse to the antigen or vaccine comprising one or more antigens, assaying for a T-helper cell response in the mouse to the antigen or vaccine comprising one or more antigens, and assaying for a T cytotoxic cell response in the mouse to the antigen or vaccine comprising one or more antigens. In some embodiments the T-helper cell response is a TH HLA-DR1 restricted response.

In some embodiments the T cytotoxic cell response is a CTRL HLA-A2 restricted response.

[028]   This invention also provides a method of optimizing two or more candidate vaccine compositions for administration to a human, based on preselected criteria. The method includes simultaneously determining the humoral response, the T-helper cell response, and the T cytotoxic cell response of a mouse following its immunization with the two or more candidate vaccine compositions, using a method comprising administering the antigen or the vaccine comprising one or more antigens to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+\beta2m°IA\beta°$, assaying for a specific humoral response in the mouse to the antigen or vaccine comprising one or more antigens, assaying for a T-helper cell response in the mouse to the antigen or vaccine comprising one or more antigens; assaying for a T cytotoxic cell response in the mouse to the antigen or vaccine comprising one or more antigens, and selecting an optimized vaccine by applying preselected criteria to the results. In some embodiments, the two or more vaccine candidates differ only in the ratio of antigen to adjuvant present in the vaccine. In some embodiments, the two or more vaccine candidates differ only in the type of adjuvant present in the vaccine.

[029]   In another aspect, the invention provides a method of determining whether a vaccine poses a risk of induction of an autoimmune disease when administered to a

human. The method comprises administering the vaccine to a transgenic mouse comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA-A2 transgene, and a functional HLA-DR1 transgene, or a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene, and has the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°, and assaying for an autoimmune response in the mouse, where observation of an autoimmune response in the mouse indicates that the vaccine poses a risk of induction of an autoimmune disease when administered to a human.

[030] This invention also provides an isolated transgenic mouse cell comprising a disrupted H2 class I gene, a disrupted H2 class II gene, and a functional HLA class I or class II transgene.

[031] In addition, the invention provides an isolated transgenic mouse cell comprising a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA class I transgene, and a functional HLA class II transgene.

[032] In some embodiments, the HLA class I transgene is an HLA-A2 transgene and the HLA class II transgene is an HLA-DR1 transgene. In other embodiments, the HLA-A2 transgene comprises the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene comprises the HLA-DR1 sequence provided in the sequence listing.

[033] Further, this invention provides an isolated transgenic mouse cell deficient for both H2 class I and class II molecules, wherein the transgenic mouse cell comprises a functional HLA class I transgene and a functional HLA class II transgene. In some

embodiments, the transgenic mouse cell has the genotype

HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°. In other embodiments, the HLA-A2 transgene comprises

the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene

comprises the HLA-DR1 sequence provided in the sequence listing.

BRIEF DESCRIPTION OF THE DRAWINGS

[034] The invention will be more fully described with reference to the drawings in

which:

[035] **Figure 1** shows a flow cytometric analysis of the cell-surface expression

of the indicated transgenic molecules. **(a)** Splenocytes from *HLA-DR1*-transgenic *H-2*

class II-KO (DR1$^+$ CII⁻, left panel), *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-

KO (A2$^\pm$ DR1$^\pm$ CI⁻ CII⁻, central panel), and *HLA-A2.1*-transgenic *H-2 class I*-KO (A2$^+$ CI⁻,

right panel) mice were stained with either FITC-labeled W6/32 (anti-HLA-ABC, in

abcissas) or biotinylated 28-8-6S (anti-H-2K^b/D^b, in ordinates) m.Ab, the latter revealed

with PE-labeled anti-mouse IgG. **(b)** B220+ splenic B lymphocytes from the same

strains of mice, were stained with FITC-labeled L243 (anti-HLA-DR1, upper panels) and

PE-labeled AF6-120.1 (anti-H-2 IAß^b, lower panels) m.Ab.

[036] **Figure 2** shows CD8$^+$ and CD4$^+$ splenic T cell numbers and *BV* segment

usage (based on an immunoscope analysis) in mice of the indicated genotypes. **(a)**

Splenocytes from *HLA-DR1*-transgenic *H-2 class II*-KO (DR1$^+$ CII⁻, left panel), *HLA-

A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-KO (A2$^+$ DR1$^+$ CI⁻ CII⁻, central panel),

and *HLA-A2.1*-transgenic *H-2 class I*-KO (A2$^+$ CI⁻, right panel) mice were stained with

PE-labeled CT-CD4 (anti-mouse CD4, in ordinates) and FITC-labeled 53-6.7 (anti-

mouse CD8, in abcissas) m.Ab. Numbers correspond to percentages of CD4$^+$ (upper left

square) or $CD8^+$ (lower right square) T cells in total splenocytes. **(b and c)** Immunoscope RT-PCR analysis of purified splenic $CD8^+$ (b) and $CD4^+$ (c) T cells for *BV* segment family (1-20) usage using forward *BV* family (1-20) specific and reverse BC primers. A typical profile for a *BV* segment family productively rearranged includes a series of peaks with a Gaussian-like distribution differing in length by 3 nucleotides. The Figure illustrates the results obtained with a *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-KO representative mouse.

[037] **Figure 3** shows HBs-specific antibody, cytolytic and proliferative responses. *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-KO mice were or not immunized by intramuscular injection of HBsAg-encoding plasmid-DNA and then individually tested. **(a)** Humoral (upper panel), cytolytic (middle panel) and proliferative (lower panel) responses and specificity controls of a representative HBsAg-DNA-immunized mouse. The antibody (IgG) titer against HBsAg particles containing both middle and small HBV envelope proteins and against the $preS2_{109-134}$ peptide were determined in an ELISA assay. Cytolytic activity at different effector/target (E/T) ratios was assessed using RMAS-HHD target cells pulsed with either relevant ($HBsAg_{348-357}$, HLA-A2.1-restricted ♦) or control ($HBsAg_{371-378}$, $H-2 K^b$-restricted Δ, and $MAGE-3_{271-279}$, HLA-A2.1- restricted □) peptide. Proliferative responses were detected using either relevant ($HBsAg_{180-195}$, HLA-DR1-restricted) or control ($HBsAg_{126-138}$, $H-2 IA^b$-restricted and HIV 1 $Gag_{263-278}$, HLA-DR1-restricted) peptide. **(b)** Similar evaluation of the antibody (IgG, upper panel), cytolytic (middle panel) and proliferative (lower panel) responses of 6 (1-6) HBsAg-DNA-immunized mice as compared to mean responses of 6 naive mice (0). Cytolytic activity at a 30/1 E/T ratio was assessed on RMAS-HHD

target cells pulsed with either HBsAg$_{348-357}$, immunodominant (filled bars) or HBsAg$_{335-343}$, subdominant (grey bars) peptide.

[038]   **Figure 4** shows results of protection assays. *HLA-A2.1-/HLA-DR1-* transgenic *H-2 class I-/class II-/*KO mice were or not immunized twice with plasmid DNA encoding HBsAg. Fifteen days after the last immunization, they were challenged intraperitoneally with 10^7 PFU of rVV expressing either the HBsAg or the HBx protein. Four days later, animals were tested individually for viral titers in ovaries. The results (rVV PFU/ovary in log 10) are given for the HBsAg-DNA-immunized mice challenged with rVV-HBsAg (I, n=10), naive mice challenged with rVV-HBsAg (N, n=6), HBsAg-immune mice challenged with rVV-HBx (Ix, n=6) and naive mice challenged with rVV-HBx (Nx, n=6).

[039]   **Figure 5** shows the AC anti-Pre S2 response in HLA-A2+DR1+CI-CII- mice following a pcmv S2/S immunization.

[040]   **Figure 6** shows the T CD4 proliferative response to HLA-DR1 restricted epitopes following immunization of HLA-A2+DR1+CI-CII- mice with pcmv S2-S.

[041]   **Figure 7** shows the T CD8 cytotoxic response to the HLA-A2 restricted HBS (348-357) peptide following an immunization of HLA-A2+DR1+CI-CII- mice with pcmv S2/S.

<div align="center">SEQUENCES</div>

[042]   SEQ ID NO:1 contains the following subparts: Nucleotides 1-1205 comprise the HLA-A2 promoter; nucleotides 1206-1265 the HLA-A2 leader sequence; nucleotides 1266-1565 the human β2 microgobulin cDNA; nucleotides 1566-1610 a (Gly4Ser)$_3$ linker; nucleotides 1611-2440 a segment containing exon 2 and part of intron

3 of HLA-A2; and nucleotides 2441-4547 a segment containing part of intron 3, exons 4 to 8, and part of the 3' non-coding region of the H_2D^b gene.

[043] SEQ ID NO:2 is the nucleotide sequence of the DRA*0101 gene. Nucleotides 1-15279 are the promoter located 5' to the HLA-DR alpha gene, nucleotides 15280-15425 are exon 1, nucleotides 15344-15346 are the ATG start codon, nucleotides 17838-18083 are exon 2, nucleotides 18575-18866 are exon 3, nucleotides 19146-19311 are exon 4, and nucleotides 20008-20340 are exon 5.

[044] SEQ ID NO:3 is the nucleotide sequence of the DRB1*010101 gene. Nucleotides 7391-7552 are exon 1, nucleotides 7453-7455 are the ATG start codon, nucleotides 15809-16079 are exon 2, nucleotides 19536-19817 are exon 3, nucleotides 20515-20624 are exon 4, nucleotides 21097-21121 are exon 5, and nucleotides 21750-22085 are exon 6.

DETAILED DESCRIPTION OF THE INVENTION

[045] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And

Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984);

the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief,

Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and

Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For

Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor

Laboratory); Immunochemical Methods In Cell And Molecular Biology (Mayer and

Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology,

Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse

Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

[046]   This invention provides mice comprising (1) mutated H-2 class I and class

II molecules; and (2) expressing HLA class I transgenic molecules, or HLA class II

transgenic molecules, or HLA class I transgenic molecules and HLA class II transgenic

molecules. Mice of the invention, which comprise a knock-out for both H-2 class I and

class II molecules, and express HLA class I transgenic molecules and HLA class II

transgenic molecules represent a completely humanized experimental mouse that can

be used to simultaneously detect the presence of antigen-specific antibodies, an

antigen-specific HLA–DRI restricted T cell response, and an antigen-specific HLA-A2

restricted T cell response. These mice are useful to study how mutual coordination

operates between a CTL response, a TH response (in particular a TH_1 or TH_2

response), and, optionally, a humoral response. These mice represent an optimized

tool for basic and applied vaccinology studies.

[047]   The invention provides transgenic mouse comprising a disrupted H2 class

I gene, a disrupted H2 class II gene, and a functional HLA class I or class II transgene.

In some embodiments, the transgenic mouse comprises a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA class I transgene, and a functional HLA class II transgene. Such a mouse can be said to be a completely humanized experimental mouse, because it can be used to simultaneously detect the presence of antigen-specific antibodies, an antigen-specific HLA–DRI restricted T cell response, and an antigen-specific HLA-A2 restricted T cell response.

[048] As shown, in part, in the Examples provided herein, and as is generally clear to one of skill in the art from the disclosure, *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-KO mice have the capacity to develop HBsAg-specific antibody, CD4$^+$ helper and CD8$^+$ cytolytic T cell responses following DNA immunization. These responses, observed in every single mouse tested, were directed at the same immunodominant epitopes as human responses and conferred to the immunized animals specific protection against a HBsAg recombinant vaccinia virus.

[049] T helper cells are essential for full maturation of antibody responses (Katz, D.H. & Benacerraf, B., *Adv Immunol* 15, 1-94 (1972)) CTL priming against many epitopes (von Boehmer, H. & Haas, W., *J Exp Med* 150, 1134-1142 (1979); Keene, J.A. & Forman, J., *J Exp Med* 155, 768-782 (1982)) and CTL long-term maintenance (Matloubian, M., Concepcion, R.J. & Ahmed, R., *J Virol* 68, 8056-8063 (1994)). Both antibodies (Lefrancois, L., *J Virol* 51, 208-214 (1984)) and CTL (Zinkernagel, R.M. & Welsh, R.M., *J Immunol* 117, 1495-1502 (1976)) are critical components of protective immunity against viral infections. Potent HBsAg-specific antibody and CTL responses were in fact observed in *HLA-A2.1-/HLA-DR1*-double transgenic, *H-2 class I-/class II*-KO mice, but not in *HLA-A2.1*-single transgenic, *H-2 class I-/ class II*-KO mice. Thus,

HBsAg-specific CD4$^+$ T cell help is essential for generating efficient HBsAg-specific CTL
and antibody responses. These results are consistent with studies on HBsAg-
immunized mice (Milich, D.R., *Semin Liver Dis* **11**, 93-112(1991)) and HBsAg-
vaccinated humans (Celis, E., Kung, P.C. & Chang, T.W., *J Immunol* **132**, 1511-1516
(1984)), which suggest that production of an anti-HBs antibody response is dependent
on CD4$^+$ T cells.

[050]   Transgenic mice expressing both HLA-A2.1 class I and HLA-DR1 class II
molecules have already been derived (BenMohamed, L. et al. *Hum Immunol* **61**, 764-
779 (2000)). The authors reported that both the HLA-A2.I and HLA-DR1 molecules are
functional restriction elements *in vivo* and that the product of the *HLA-DR1* transgene
enhances the HLA-A2.1-restricted antigen-specific CTL responses. However, the
human relevance of the immune responses in these mice is dwarfed by the fact that
they still expressed their own H-2 class I and class II molecules, which are usually
preferentially and often exclusively used as restricting elements in response to antigens
(Ureta-Vidal, A., Firat, H., Perarnau, B. & Lemonnier, F.A., *J Immunol* **163**, 2555-2560
(1999); Rohrlich, P.S. et al., *Int Immunol* **15**, 765-772 (2003)) (A. Pajot, unpublished
results). The invention described herein overcomes this limitation by providing *HLA-
A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mice.

[051]   In some embodiments the *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-
/class II*-KO mice express, in a *β2m*-KO context, a HLA-A2.1 monochain in which the
human $β2m$ is covalently linked by a peptidic arm to the HLA-A2.1 heavy chain. They
further lack cell surface expression of conventional H-2 IA and IE class II molecules as
a result of the inactivation of the *H-2 IAβ^b* gene, since *H-2 IEα* is a pseudogene in the H-

2^b haplotype. The results provided herein demonstrate that such mice are deprived of cell surface expression of H-2 class I and class II molecules. However, it was reported in one case that a free class I heavy chain, in particular H-2 D^b, may exist on the surface of a $\beta 2m$-KO mouse, and could induce an alloreactivity response. Even if this is so, because such mice are empty of peptide, they should not interfere in antigen-specific immune response (Bix, M. & Raulet, D., J Exp Med 176, 829-834 (1992)). This is supported by the report of Allen et al (Allen, H., Fraser, J., Flyer, D., Calvin, S. & Flavell, R., Proc Natl Acad Sci U S A 83, 7447-7451 (1986)), in which they confirmed that H-2 D^b is expressed at the cell surface even when there is no $\beta 2m$ present within the cell, but that such D^b antigen is recognized by neither D^b-allospecific or D^b-restricted cytotoxic T lymphocytes. Furthermore, D^b antigens are not recognized by most monoclonal antibodies of the native D^b.

[052] Nonetheless, in HLA-DRa single transgenic mice, it was reported that unconventional HLA-DRa/H-2 $IE\beta^b$ hybrid complexes may be expressed to some extent on the cell surface, at least in the absence of the HLA-DRβ chain (Lawrance, S.K. et al., Cell 58, 583-594 (1989)). In spite of this observation, these unconventional molecules were not detected serologically on cell surfaces in HLA-A2.1-/HLA-DR1-transgenic, H-2 class I-/class II-KO mice, even with mAb (17-3-3S), which is known to react with such hybrid molecules (Ozato, K., Mayer, N. & Sachs, D.H., J Immunol 124, 533-540 (1980)) (Figure 1a and data not shown). In addition, the results obtained on studying HBsAg-specific and HIV 1-Gag-specific T cell responses of these mice were all indicative of exclusive usage of the HLA-A2.1 and HLA-DR1 molecules as restricting elements. This argues that the unconventional HLA-DRa/H-2 $IE\beta^b$ hybrids were likely unstable

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compared to conventional *HLA-DRα/*HLA-DRβ molecules and that they may exist only

in the absence of the HLA-DRβ chain. Mouse strains in which the entire (H-2 $IA\beta^b$,

$IA\alpha^b$, $IE\beta^b$) H-2 class II region has been deleted (Madsen, L. et al., *Proc Natl Acad Sci U*

S A **96**, 10338-10343 (1999)), as well as the H-2 D^b gene, are being analyzed to

completely exclude this possibility. Preliminary analysis of splenocytes obtained from

the first animals revealed a CD4$^+$ T cell pool restoration similar to that observed in *HLA-*

DR1-transgenic *H-2 class II*-KO ($Ia\beta^{b\cdot}$) mice, suggesting that the *HLA-DR1*-restricted

CD4$^+$ T cell responses of these new mice should be equivalent to those of the *HLA-*

A2.1-/*HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mice.

[053] The peripheral CD8$^+$ T lymphocytes of *HLA-A2.1*-/*HLA-DR1*-transgenic,

H-2 class I-/class II-KO mice, compared to parental *HLA-A2.1*-transgenic *H-2 class I*-KO

mice, are quantitatively and qualitatively similar with full diversification, at least in terms

of *BV* segment usage, of the TCR repertoire. Partial restoration compared to wild-type

animals, especially of the CD8$^+$ T cell pool, has been a constant observation in single

HLA-transgenic mice expressing a chimeric ($\alpha3$ domain of mouse origin) HLA-A2.1

molecule (Pascolo, S. et al., *J Exp Med* **185**, 2043-2051 (1997)). Regardless of the $\alpha3$

domain substitution, the interaction remains suboptimal between mouse CD8 and HLA-

A2.1 molecules, since co-crystal analysis has documented that human CD8 also

contacts the HLA-A2.1 heavy chain $\alpha2$ domain (Gao, G.F. et al., *Nature* **387**, 630-634

(1997)). Suboptimal cooperation might also occur in the endoplasmic reticulum where

many molecules (TAP, tapasine, ERp 57) assist MHC class I molecule biosynthesis.

However, at this stage, the only documented functional difference between these mice

and human endoplasmic reticulum molecules, namely the efficient transport by human

but not mouse TAP of COOH-terminus positively charged cytosolic peptides (Momburg, F., Neefjes, J.J. & Hammerling, G.J., *Curr Opin Immunol* 6, 32-37 (1994)), is not relevant for HLA-A2.1 molecules which bind peptides with a hydrophobic C-terminus, since these peptides are transported efficiently by mouse and human TAP. Even though the number of CD8^{+} T lymphocytes is lower in both single *HLA-A2.1*-transgenic, *H-2 class I*-KO mice and in *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-KO mice, they respond efficiently against HBsAg and, importantly, the latter mice develop antibody, helper and cytolytic cell responses similar to humans.

[054] One of the difficulties hampering the design of T-epitope-based vaccines targeting T lymphocytes is HLA class I/class II molecule polymorphism. HLA-A2.1 and HLA-DR1 molecules are expressed by a significant proportion of individuals in human populations (30 to 50% for HLA-A2.1, 6 to 18 % for HLA-DR1). Even though the functional clustering of HLA class I molecules in superfamilies is based on significant redundancy of the presented sets of peptides[34], individual analysis of the responses elicited by each HLA class I isotypic or allelic variant remains desirable to identify the optimal epitopes they present: This is particularly important to devise a new reagent, such as tetramer (HLA-class I or HLA-class II) to monitor the immune response. For the same reason, it would be helpful to obtain strains of mice co-expressing HLA-A2.1 with other HLA class II molecules, even if the binding of peptides to HLA class II molecules is less restrictive than to class I molecules. Based on the disclosure herein, additional HLA class I-/class II-transgenic, H-2 class I-/class II-KO mice can be constructed for these and other purposes.

[055]   Whereas *HLA*-transgenic *H-2*-KO mice enable a detailed analysis and

optimization of the immunogenicity of antigenic peptides with excellent transposability to

humans (Rohrlich, P.S. et al., *Int Immunol* **15**, 765-772 (2003); Loirat, D., Lemonnier,

F.A. & Michel, M.L., *J Immunol* **165**, 4748-4755 (2000); Scardino, A. et al., *Eur J*

Immunol **31**, 3261-3270 (2001)) this is less evident for vaccine adjuvant-formulation

studies. This could be due to differences between the two species in the various

effectors that are mobilized early in response to an antigenic challenge. Increasing

fundamental knowledge of innate immunity might, in the future, lead to a more complete

humanization of the mouse immune system.

[056]   In conclusion, the disclosure herein describes an optimized, humanized

transgenic mouse model, whose *H-2 class I* (*mouse β2m*) and *class II* (*H-2 IAβ^{b}*) genes

have been deleted and replaced with equivalent human genes *HHD* (*HLA-A*0201*),

*HLA-DRA*0101* and *HLA-DRB1*0101*. Cellular immunity in the *HLA-A2.1-/HLA-DR1-*

transgenic *H-2 class I-/class II*-KO mice is completely restricted by the human HLA

molecules, with a complete absence of immune responses restricted by the murine

MHC molecules. The absence of competition between murine MHC and human

(transgenic) HLA immune responses allows for use of these mice to characterize

epitopes in human vaccines that require collaboration between HLA-restricted CD4^{+} T

helper and HLA-restricted CD8^{+} T cytolytic cells.

[057]   "HLA" is the human MHC complex, and "H-2" the mouse MHC complex.

The human complex comprises three class I *α*-chain genes, HLA-A, HLA-B, and HLA-C,

and three pairs of MHC class II *α*- and *β*-chain genes, HLA-DR, -DP, and -DQ. In many

haplotypes, the HLA-DR cluster contains an extra *β*-chain gene whose product can pair

with the DRα chain, and so the three sets of genes give rise to four types of MHC class II molecules. In the mouse, the three class I α-chain genes are H-2-L, H-2-D, and H-2-K. The mouse MHC class II genes are H-2-A and H-2-E.

[058] It is known in the art that genetic diversity exists between the HLA genes of different individuals as a result of both polymorphic HLA antigens and distinct HLA alleles. Accordingly, embodiments of the invention disclosed herein may substitute one polymorphic HLA antigen for another or one HLA allele for another. Examples of HLA polymorphisms and alleles can be found, for example, at http://www.anthonynolan.org.uk/HIG/data.html and http://www.ebl.ac.uk/imgt/hla, and in Genetic diversity of HLA: Functional and Medical Implication, Dominique Charon (Ed.), EDK Medical and Scientific International Publisher, and The HLA FactsBook, Steven G.E. Marsh, Peter Parham and Linda Barber, AP Academic Press, 2000.

[059] A "disrupted" gene is one that has been mutated using homologous recombination or other approaches known in the art. A disrupted gene can be either a hypomorphic allele of the gene or a null allele of the gene. One of skill in the art will recognize that the type of allele to be used can be selected for any particular context. In many embodiments of the invention, a null allele is preferred.

[060] "Homologous recombination" is a general approach for targeting mutations to a preselected, desired gene sequence of a cell in order to produce a transgenic animal (Mansour, S. L. et al., *Nature* 336:348-352 (1988); Capecchi, M. R., *Trends Genet.* 5:70-76 (1989); Capecchi, M. R., *Science* 244:1288-1292 (1989); Capecchi, M. R. et al., In: Current Communications in Molecular Biology, Capecchi, M. R. (ed.), Cold

Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), pp. 45-52; Frohman, M. A. et al., *Cell* 56:145-147 (1989)).

[061]   It is now be feasible to deliberately alter any gene in a mouse (Capecchi, M. R., *Trends Genet.* 5:70-76 (1989); Frohman, M. A. et al., *Cell* 56:145-147 (1989)). Gene targeting involves the use of standard recombinant DNA techniques to introduce a desired mutation into a cloned DNA sequence of a chosen locus. That mutation is then transferred through homologous recombination to the genome of a pluripotent, embryo-derived stem (ES) cell. The altered stem cells are microinjected into mouse blastocysts and are incorporated into the developing mouse embryo to ultimately develop into chimeric animals. In some cases, germ line cells of the chimeric animals will be derived from the genetically altered ES cells, and the mutant genotypes can be transmitted through breeding.

[062]   Gene targeting has been used to produce chimeric and transgenic mice in which an nptII gene has been inserted into the β_2 -microglobulin locus (Koller, B. H. et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 86:8932-8935 (1989); Zijlstra, M. et al., *Nature* 342:435-438 (1989); Zijlstra, M. et al., *Nature* 344:742-746 (1989); DeChiaba et al., *Nature* 345:78-80 (1990)). Similar experiments have enabled the production of chimeric and transgenic animals having a c-abl gene which has been disrupted by the insertion of an nptII gene (Schwartzberg, P. L. et al., *Science* 246:799-803 (1989)). The technique has been used to produce chimeric mice in which the en-2 gene has been disrupted by the insertion of an nptII gene (Joyner, A. L. et al., *Nature* 338:153-155 (1989)).

[063] In order to utilize the "gene targeting" method, the gene of interest must have been previously cloned, and the intron-exon boundaries determined. The method results in the insertion of a marker gene (e.g., an nptII gene) into a translated region of a particular gene of interest. Thus, use of the gene targeting method results in the gross destruction of the gene of interest.

[064] Significantly, the use of gene targeting to alter a gene of a cell results in the formation of a gross alteration in the sequence of that gene. The efficiency of gene targeting depends upon a number of variables, and is different from construct to construct.

[065] The chimeric or transgenic animal cells of the present invention are prepared by introducing one or more DNA molecules into a cell, which may be a precursor pluripotent cell, such as an ES cell, or equivalent (Robertson, E. J., In: Current Communications in Molecular Biology, Capecchi, M. R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), pp. 39-44). The term "precursor" is intended to denote only that the pluripotent cell is a precursor to the desired ("transfected") pluripotent cell, which is prepared in accordance with the teachings of the present invention. The pluripotent (precursor or transfected) cell can be cultured in vivo in a manner known in the art (Evans, M. J. et al., Nature 292:154-156 (1981)) to form a chimeric or transgenic animal.

[066] Any ES cell can be used in accordance with the present invention. It is, however, preferred to use primary isolates of ES cells. Such isolates can be obtained directly from embryos, such as the CCE cell line disclosed by Robertson, E. J., In: Current Communications in Molecular Biology, Capecchi, M. R. (ed.), Cold Spring

Harbor Press, Cold Spring Harbor, N.Y. (1989), pp. 39-44), or from the clonal isolation

of ES cells from the CCE cell line (Schwartzberg, P. A. et al., Science 246:799-803

(1989), which reference is incorporated herein by reference). Such clonal isolation can

be accomplished according to the method of E. J. Robertson (In: Teratocarcinomas and

Embryonic Stem Cells: A Practical Approach, (E. J. Robertson, Ed.), IRL Press, Oxford,

1987), which reference and method are incorporated herein by reference. The purpose

of such clonal propagation is to obtain ES cells, which have a greater efficiency for

differentiating into an animal. Clonally selected ES cells are approximately 10-fold more

effective in producing transgenic animals than the progenitor cell line CCE. For the

purposes of the recombination methods of the present invention, clonal selection

provides no advantage.

[067] An example of ES cell lines, which have been clonally derived from

embryos, are the ES cell lines, AB1 (hprt$^+$) or AB2.1 (hprt$^-$). The ES cells are preferably

cultured on stromal cells (such as STO cells (especially SNC4 STO cells) and/or

primary embryonic fibroblast cells) as described by E. J. Robertson (In:

Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E. J. Robertson,

Ed., IRL Press, Oxford, 1987, pp 71-112), which reference is incorporated herein by

reference. Methods for the production and analysis of chimeric mice are disclosed by

Bradley, A. (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E.

J. Robertson, Ed.), IRL Press, Oxford, 1987, pp 113-151), which reference is

incorporated herein by reference. The stromal (and/or fibroblast) cells serve to

eliminate the clonal overgrowth of abnormal ES cells. Most preferably, the cells are

cultured in the presence of leukocyte inhibitory factor ("lif") (Gough, N. M. et al., *Reprod.*

Fertil. Dev. 1:281-288 (1989); Yamamori, Y. et al., Science 246:1412-1416 (1989), both

of which references are incorporated herein by reference). Since the gene encoding lif

has been cloned (Gough, N. M. et al., Reprod. Fertil. Dev. 1:281-288 (1989)), it is

especially preferred to transform stromal cells with this gene, by means known in the

art, and to then culture the ES cells on transformed stromal cells that secrete lif into the

culture medium.

[068] As used herein, the term "transgene" refers to a nucleic acid sequence,

which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into

which it is introduced, or, is homologous to an endogenous gene of the transgenic

animal or cell into which it is introduced, but which is designed to be inserted, or is

inserted, into the animal's genome in such a way as to alter the genome of the cell into

which it is inserted (e.g., it is inserted at a location which differs from that of the natural

gene or its insertion results in a knockout). A transgene can be operably linked to one

or more transcriptional regulatory sequences and any other nucleic acid, such as

introns, that may be necessary for optimal expression of a selected nucleic acid.

Exemplary transgenes of the present invention encode, for instance an H-2 polypeptide.

Other exemplary transgenes are directed to disrupting one or more HLA genes by

homologous recombination with genomic sequences of an HLA gene.

[069] A "functional transgene" is one that produces an mRNA transcript, which

in turn produces a properly processed protein in at least one cell of the mouse

comprising the transgene. One of skill will realize that the diverse set of known

transcriptional regulatory elements and sequences directing posttranscriptional

processing provide a library of options from which to direct the expression of a

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transgene is a host mouse. In many embodiments of the invention, expression of an HLA transgene under the control of an H-2 gene regulatory element may be preferred.

[070] In some embodiments, the HLA class I transgene is an HLA-A2 transgene and the HLA class II transgene is an HLA-DR1 transgene. An example of an HLA-A2 transgene is one that comprises the HLA-A2 sequence provided in the sequence listing. An example of an HLA-DR1 transgene is one that comprises the HLA-DR1 sequence provided in the sequence listing.

[071] In an embodiment, the invention provides a transgenic mouse deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene. In some embodiments, the mouse has the genotype HLA-A2$^+$HLA-DR1$^+$β2m^{o}IAβ^o. In other embodiments the HLA-A2 transgene comprises the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene comprises the HLA-DR1 sequence provided in the sequence listing.

[072] The invention also provides isolated transgenic mouse cells. In some cases the cell comprises a disrupted H2 class I gene, a disrupted H2 class II gene, and a functional HLA class I or class II transgene. In others, the cell comprises a disrupted H2 class I gene, a disrupted H2 class II gene, a functional HLA class I transgene, and a functional HLA class II transgene. The HLA class I transgene can be an HLA-A2 transgene and the HLA class II transgene can be an HLA-DR1 transgene. In some cases, the HLA-A2 transgene comprises the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene comprises the HLA-DR1 sequence provided in the sequence listing.

[073] In an embodiment, the invention provides an isolated transgenic mouse cell deficient for both H2 class I and class II molecules, wherein the transgenic mouse comprises a functional HLA class I transgene and a functional HLA class II transgene. The isolated transgenic mouse cells can have the genotype HLA-A2$^+$HLA-DR1$^+$β2m°IAβ°. The HLA-A2 transgene can comprise the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene can comprise the HLA-DR1 sequence provided in the sequence listing.

[074] The isolated transgenic mouse cells of the invention can have the genotype of any mouse of the invention. However, the set of genotypes of the isolated transgenic mouse cells of the invention, and the set of genotypes of the mice of the invention are not necessarily entirely overlapping.

[075] The isolated mouse cells of the invention can be obtained from a mouse or mouse embryo. In one embodiment, the mouse or mouse embryo has the same genotype as the cell to be obtained. In another embodiment, the mouse or mouse embryo has a different genotype than the cell to be obtained. After the cell is obtained from the mouse or mouse embryo, a gene of the cell can be disrupted by, for example, homologous recombination. Additionally, a functional transgene can be introduced into the genome of the cell by, for example, transfection. One of skill in the art will recognize that any suitable method known in the art can be applied to modify the genome of the cell to thereby obtain an isolated mouse cell having the desired genotype.

[076] An additional object of the invention is an isolated transgenic mouse cell deficient for both H2 class I and class II molecules, wherein the transgenic mouse cell comprises a functional HLA class I transgene and a functional HLA class II transgene.

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In some embodiments, the transgenic mouse cell has the genotype

HLA-A2$^+$HLA-DR1$^+\beta$2m°IAβ°. In other embodiments, the HLA-A2 transgene comprises

the HLA-A2 sequence provided in the sequence listing and the HLA-DR1 transgene

comprises the HLA-DR1 sequence provided in the sequence listing.

[077] T cells play a central role in many aspects of acquired immunity, carrying

out a variety of regulatory and defensive functions. When some T cells encounter an

infected or cancerous cell, they recognize it as foreign and respond by acting as killer

cells, killing the host's own cells as part of the cell-mediated immune response. Other T

cells, designated helper T cells, respond to perceived foreign antigens by stimulating B

cells to produce antibodies, or by suppressing certain aspects of a humoral or cellular

immune response.

[078] T helper cells (Th) orchestrate much of the immune response via the

production of cytokines. Although generally identifiable as bearing the CD4 cell surface

marker, these cells are functionally divided into Th1 or Th2 subpopulations according to

the profile of cytokines they produce and their effect on other cells of the immune

system.

[079] The Th1 cells detect invading pathogens or cancerous host cells through a

recognition system referred to as the T cell antigen receptor. Termed cellular

immunity, Th1-related processes generally involve the activation of non-B cells and are

frequently characterized by the production of IFN-γ. Nevertheless, although the Th1

system is primarily independent from the production of humoral antibodies, Th1

cytokines do promote immunoglobulin class switching to the IgG$_{2a}$ isotype.

[080] Upon detection of a foreign antigen, most mature Th1 cells direct the release of IL-2, IL-3, IFN-γ, TNF-β, GM-CSF, high levels of TNF-α, MIP-1α, MIP-1β, and RANTES. These cytokines promote delayed-type hypersensitivity and general cell-mediated immunity. IL-2, for instance, is a T cell growth factor that promotes the production of a clone of additional T cells sensitive to the particular antigen that was initially detected. The sensitized T cells attach to and attack cells or pathogens containing the antigen.

[081] In contrast, mature Th2 cells tend to promote the secretion of IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF, and low levels of TNF-α. In addition, the Th2 response promotes humoral immunity by activating B cells, stimulating antibody production and secretion, and inducing class switching to IgA, IgG$_1$ and IgE isotypes.

[082] As used herein, an "antigen" comprises: 1) at least one HTL epitope, or 2) at least one CTL epitope or, 3) at least one B cell epitope, or 4) at least one HTL epitope and at least one CTL epitope, or 5) at least one HTL epitope and at least one B cell epitope, or 6) at least one CTL epitope and at least one B cell epitope, or 7) at least one HTL epitope and at least one CTL epitope and at least one B cell epitope. A "candidate antigen" is a molecule that is under investigation to determine whether it functions as an antigen.

[083] A "humoral immune response" is antibody-mediated specific immunity.

[084] An "epitope" is a site on an antigen that is recognized by the immune system. An antibody epitope is a site on an antigen recognized by an antibody. A T-cell epitope is a site on an antigen that binds to an MHC molecule. A TH epitope is one

that binds to an MHC class II molecule. A CTL epitope is one that binds to an MHC class I molecule.

[085] The antigen can comprise a polypeptide sequence or a polynucleotide sequence, which can comprise RNA, DNA, or both. In one embodiment, the antigen comprises at least one polynucleotide sequence operationally encoding one or more antigenic polypeptides. Used in this context, the word "comprises" intends that at least one antigenic polypeptide is provided by the transcription and/or translation apparatus of a host cell acting upon an exogenous polynucleotide that encodes at least one antigenic polypeptide, as described, for example in U.S. Patent No. 6,194,389 and 6,214,808.

[086] Antigens of the invention can be any antigenic molecule. Antigenic molecules include: proteins, lipoproteins, and glycoproteins, including viral, bacterial, parasitic, animal, and fungal proteins such as albumins, tetanus toxoid, diphtheria toxoid, pertussis toxoid, bacterial outer membrane proteins (including meningococcal outer membrane protein), RSV-F protein, malarial derived peptide, B-lactoglobulin B, aprotinin, ovalbumin, lysozyme, and tumor associated antigens such as carcinoembryonic antigen (CEA), CA 15-3, CA 125, CA 19-9, prostrate specific antigen (PSA), and the TAA complexes of U.S. Patent No. 5,478,556, which is incorporated herein by reference in its entirety; carbohydrates, including naturally-occurring and synthetic polysaccharides and other polymers such as ficoll, dextran, carboxymethyl cellulose, agarose, polyacrylamide and other acrylic resins, poly (lactide-co-glycolide), polyvinyl alcohol, partially hydrolyzed polyvinyl acetate, polyvinylpyrolidine, Group B Steptococcal and Pneumococcal capsular polysaccharides (including type III),

Pseudomonas aeruginosa mucoexopolysaccharide, and capsular polysaccharides (including fisher type I), and *Haemophilus influenzae* polysaccharides (including PRP); haptens, and other moieties comprising low molecular weight molecules, such as TNP, saccharides, oligosaccharides, polysaccharides, peptides, toxins, drugs, chemicals, and allergens; and haptens and antigens derived from bacteria, rickettsiae, fungi, viruses, parasites, including Diphtheria, Pertussis, Tetanus, *H. influenzae*, *S. pneumoniae*, *E. Coli*, Klebsiella, *S. aureus*, *S. epidermidis*, *N. meningiditis*, Polio, Mumps, measles, rubella, Respiratory Syncytial Virus, Rabies, Ebola, Anthrax, Listeria, Hepatitis A, B, C, Human Immunodeficiency Virus I and II, Herpes simplex types 1 and 2, CMV, EBV, Varicella Zoster, Malaria, Tuberculosis, *Candida albicans*, and other candida, *Pneumocystis carinii*, Mycoplasma, Influenzae virus A and B, Adenovirus, Group A streptococcus, Group B streptococcus, *Pseudomonas aeryinosa*, Rhinovirus, Leishmania, Parainfluenzae, types 1, 2 and 3, Coronaviruses, Salmonella, Shigella, Rotavirus, Toxoplasma, Enterovirusses, and *Chlamydia trachomatis* and *pneumoniae.*

[087] As used herein, a pharmaceutical composition or vaccine comprises at least one immunological composition, which can be dissolved, suspended, or otherwise associated with a pharmaceutically acceptable carrier or vehicle. Any pharmaceutically acceptable carrier can be employed for administration of the composition. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, 18th Edition (A. Gennaro, ed., 1990) Mack Pub., Easton, Pa., which is incorporated herein by reference in its entirety. Carriers can be sterile liquids, such as water, polyethylene glycol, dimethyl sulfoxide (DMSO), oils, including petroleum oil, animal oil, vegetable oil, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Carriers can be in the form

of mists, sprays, powders, waxes, creams, suppositories, implants, salves, ointments, patches, poultices, films, or cosmetic preparations.

[088]   Proper formulation of the pharmaceutical composition or vaccine is dependent on the route of administration chosen.   For example, with intravenous administration by bolus injection or continuous infusion, the compositions are preferably water soluble, and saline is a preferred carrier. For transcutaneous, intranasal, oral, gastric, intravaginal, intrarectal, or other transmucosal administration, penetrants appropriate to the barrier to be permeated can be included in the formulation and are known in the art. For oral administration, the active ingredient can be combined with carriers suitable for inclusion into tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like. Time-sensitive delivery systems are also applicable for the administration of the compositions of the invention. Representative systems include polymer base systems, such as poly(lactide-glycoside), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid and polyanhydrides. These and like polymers can be formulated into microcapsules according to methods known in the art, for example, as taught in U.S. Patent No. 5,075,109, which is incorporated herein by reference in its entirety. Alternative delivery systems appropriate for the administration of the disclosed immunostimulatory compounds of the invention include those disclosed in U.S. Patents No. 6,194,389, 6,024,983 5,817,637, 6,228,621, 5,804,212, 5,709,879, 5,703,055, 5,643,605, 5,643,574, 5,580,563, 5,239,660, 5,204,253, 4,748,043, 4,667,014, 4,452,775, 3,854,480, and 3,832,252 (each of which is incorporated herein by reference in its entirety).

[089] Aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable or aerosol solutions. For administration by aerosol, as by pressurized spray or nebulizer, suitable propellants can be added as understood by those familiar with the art. The immunological composition can also be formulated with solubilizing agents; emulsifiers; stabilizers; dispersants; flavorants; adjuvants; carriers; topical anesthetics, such as lidocaine, xylocaine, and the like; antibiotics; and known or suspected anti-viral, anti-fungal, anti-parasitic, or anti-tumor compounds.

[090] An "adjuvant" is a composition that promotes or enhances an immune response to a target antigen. One of skill in the art can select an appropriate adjuvant for use in practicing the present invention in view of the disclosure herein.

[091] The present invention encompasses methods of treating a patient in need of immune stimulation by administering a composition comprising one or more antigens of the invention. As used herein, treatment encompasses corrective, restorative, ameliorative, and preventive methods relating to any disease, condition, abnormality, or symptom. Treatment further encompasses the elicitation or suppression of an immune response in an experimental animal or *ex vivo*.

[092] Thus, treatment comprises administering an immunostimulatory amount of any of the immunostimulatory compositions of the invention by any method familiar to those of ordinary skill in the art, commonly including oral and intranasal routes, and intravenous, intramuscular, and subcutaneous injections, but also encompassing, intraperitoneal, intracorporeal, intra-articular, intraventricular, intrathecal, topical, tonsillar, mucosal, transdermal, intravaginal administration and by gavage.

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[093] As is recognized by the skilled practitioner, choosing an appropriate administration method may contribute to the efficacy of a treatment, and local administration may be preferred for some applications. Acceptable routes of local administration include subcutaneous, intradermal, intraperitoneal, intravitreal, inhalation or lavage, oral, intranasal, and directed injection into a predetermined tissue, organ, joint, tumor, or cell mass. For example, mucosal application or injection into mucosal lymph nodes or Peyer's patches may promote a humoral immune response with substantial IgA class switching. Alternatively, targeted injection into a lesion, focus, or affected body site may be applicable for the treatment of solid tumors, localized infections, or other situs requiring immune stimulation.

[094] Alternatively, cells of the immune system (e.g., T cells, B cells, NK cells, or oligodendrocytes) can be removed from a host and treated *in vitro*. The treated cells can be further cultured or reintroduced to a patient (or to a heterologous host) to provide immune stimulation to the patient or host. For example, bone marrow cells can be aspirated from a patient and treated with an HDR to stimulate global or specific immunity. High-dose radiation, or comparable treatments, can then be used to destroy the remaining immune cells in the patient. Upon re-implantation, the autologous stimulated cells will restore normal immune function in the patient. Alternatively, NK and/or T cells isolated from a patient suffering from cancer may be exposed *in vitro* to one or more antigens specific to the patient's cancer. Upon re-implantation into the patient, the antigen-stimulated cells will deploy a vigorous cellular immune response against the cancerous cells.

[095] An immunostimulatory (efficacious) amount refers to that amount of vaccine that is able to stimulate an immune response in a patient, which is sufficient to prevent, ameliorate, or otherwise treat a pathogenic challenge, allergy, or immunologic abnormality or condition. An immunostimulatory amount is that amount, which provides a measurable increase in a humoral or cellular immune response to at least one epitope of the antigen as compared to the response obtained if the antigen is administered to the patient without prior treatment with the vaccine. Thus, for example, an immunostimulatory amount refers to that amount of an antigen-containing composition that is able to promote the production of antibodies directed against an antigenic epitope of interest or stimulate a detectable protective effect against a pathogenic or allergenic challenge or to promote a protective CTL response against an antigenic epitope of interest.

[096] Treatment with an immunostimulatory amount of an antigen-containing composition of the invention comprises effecting any directly, indirectly, or statistically observable or measurable increase or other desired change in the immune response in a host, specifically including an *ex vivo* tissue culture host, comprising at least one cell of the immune system or cell line derived therefrom. Host cells can be derived from human or animal peripheral blood, lymph nodes or the like. Preferred tissue culture hosts include freshly isolated T cells, B cells, macrophages, oligodendrocytes, NK cells, and monocytes, each of which can be isolated or purified using standard techniques. Observable or measurable responses include, B or T cell proliferation or activation; increased antibody secretion; isotype switching; increased cytokine release, particularly the increased release of one or more of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-

12, IL-13, GM-CSF, IFN-γ, TNF-α, TNF-β, GM-CSF, MIP-1α, MIP-1β, or RANTES; increased antibody titer or avidity against a specific antigen; reduced morbidity or mortality rates associated with a pathogenic infection; promoting, inducing, maintaining, or reinforcing viral latency; suppressing or otherwise ameliorating the growth, metastasis, or effects of malignant and non-malignant tumors; and providing prophylactic protection from a disease or the effects of a disease.

[097]   Where the suppression of an immunological response is desired, for example, in the treatment of autoimmune disease or allergy, an effective amount also encompasses that amount sufficient to effect a measurable or observable decrease in a response associated with the condition or pathology to be treated.

[098]   The amount of an antigen-containing composition to be administered and the frequency of administration can be determined empirically and will take into consideration the age and size of the patient being treated, and the condition or disease to be addressed. An appropriate dose is within the range of 0.01 μg to 100 μg per inoculum, but higher and lower amounts may also be indicated. Secondary booster immunizations can be given at intervals ranging from one week to many months later.

[099]   The following examples demonstrate certain embodiments of the invention. One of ordinary skill in the art will recognize the numerous modifications and variations that may be performed without altering the spirit or scope of the present invention. Such modifications and variations are believed to be encompassed within the scope of the invention. The examples do not in any way limit the invention.

EXAMPLES

[0100] The following experimental techniques and reagents were used to demonstrate certain nonlimiting embodiments of the invention.

[0101] <u>Transgenic Mice</u>

[0102] The *HLA-DR1*-transgenic *H-2 class II*-KO (*IA β^b*) mice were obtained at the Institut Pasteur of Lille by crossing *HLA-DR1*-transgenic mice (Altmann, D.M. et al., *J Exp Med* **181**, 867-875 (1995)) with *H-2 class II*-KO (*IA β^b*) mice (Rohrlich, P.S. et al., *Int Immunol* **15**, 765-772 (2003)). The *HLA-A2.1*-transgenic mice, expressing a chimeric monochain (HHD molecule : α1-α2 domains of HLA-A2.1, α3 to cytoplasmic domains of H-2 D^b, linked at its N-terminus to the C terminus of human β2m by a 15 amino-acid peptide linker) were created (Pascolo, S. et al., *J Exp Med* **185**, 2043-2051 (1997)). *HLA-A2.1 (HHD)*-transgenic *H-2 class I*-KO and *HLA-DR1*-transgenic *H-2 class II*-KO (*IA β^b*) mice were intercrossed and progenies screened until *HLA-A2.1$^{+/-}$* /*HLA-DR1$^{+/-}$* double transgenic *H-2-class I (β2m^0)-/ class II (IAβ^0)*-KO animals were obtained and used for the experiments described herein . *HLA-A2.1$^{+/-}$* single transgenic *H-2-class I (β2m^0)-/ class II (IAβ^0)*-KO mice were used as controls in the protection assays. Mice were bred in the animal facilities at the Institut Pasteur, Paris; all protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations on Animal Welfare and with Public Health Service recommendations.

[0103] <u>Genotyping</u>

[0104] The *HLA-DRB1*0101, HLA-DRA*0101* and *HLA-A*0201* transgenes were detected by PCR. Tail-DNA was extracted after overnight incubation at 56°C in 100 mM

NaCl, 50 mM Tris-HCl pH 7.2, 100 mM EDTA, 1 % SDS and 0.5 mg/ml proteinase K,

followed by the addition of 250 μl of saturated NaCl solution and isopropanol

precipitation. The samples were washed (3x) in 70 % ethanol and resuspended in 150

μl of 10 mM Tris-HCl, 1 mM EDTA pH 8. PCR conditions were: 1.5 mM $MgCl_2$, 1.25 U

of Taq Polymerase, buffer supplied by the manufacturer (InVitrogen, Carlsbad, CA), 1

cycle (7 min, 94 °C), 40 cycles (30 sec, 94 °C; 30 sec, 60 °C; 1 min, 72 °C), 1 cycle (4

min, 72°C), using as forward and reverse primers, for *HHD* : 5'CAT TGA GAC AGA

GCG CTT GGC ACA GAA GCA G 3' and 5'GGA TGA CGT GAG TAA ACC TGA ATC

TTT GGA GTA CGC 3', for *HLA-DRB1*0101* : 5' TTC TTC AAC GGG ACG GAG CGG

GTG 3' and 5' CTG CAC TGT GAA GCT CTC ACC AAC 3', and for *HLA-DRA*0101* : 5'

CTC CAA GCC CTC TCC CAG AG 3' and 5' ATG TGC CTT ACA GAG GCC CC 3'.

[0105] <u>FACS analysis</u>

[0106] Cytofluorimetry studies were performed on red-blood cell-depleted,

Lympholyte M-purified (Tebu-bio, Le Perray en Yvelines, France) splenocytes using

FITC-conjugated W6/32 (anti-HLA-ABC, Sigma, St Louis, MO) and biotinilated anti-28-

8-6S (anti-H-2 K^b/D^b, BD Biosciences, San Diego, CA) m.Ab. $CD4^+$ and $CD8^+$ T

lymphocytes were stained using PE-labeled CT-CD4 anti-mouse CD4 (CALTAG, South

San Francisco, CA) and FITC-labeled 53-6.7 anti-mouse CD8 m.Ab (BD Biosciences).

Analysis of MHC class II molecule expression was performed on $B220^+$ B lymphocytes

positively selected on MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

Following saturation of Fc receptors with 2.4G2 m.Ab, expression of HLA-DR1 and H-2

IA^b was analyzed using FITC-labeled L243 (anti-HLA-DR) and PE-labeled AF6-120.1

(anti-H-2 IAβ^b) m.Ab (BD Biosciences). Paraformaldehyde fixed cells were analyzed with a FACSCalibur (Becton Dickinson, Bedford, MA).

[0107] Immunoscope analyses

[0108] CD4$^+$ and CD8$^+$ T cells from naive mice were positively selected on Auto-Macs (Miltenyi Biotec), RNA prepared using RNA Easy Kit (Qiagen, Hilden, Germany) and used for cDNA synthesis. The cDNA was PCR-amplified using forward primers specific for each BV segment family and a reverse primer shared by the two BC segments. PCR-products were subjected to a run-off-elongation with internal BC FAM-tagged primer. The run-off products were loaded on a 6% acrylamide/8 M urea gel for separation (7 h, 35 W) with a 373A DNA sequencer (Perkin Elmer Applied Biosystem, Foster City, CA). Data were analyzed using immunoscope software (Pannetier, C. et al., *Proc Natl Acad Sci U S A* **90**, 4319-4323 (1993)).

[0109] Peptides

[0110] The HLA-A2 binding peptides HBsAg$_{348-357}$ GLSPTVWLSV and HBsAg$_{335-343}$ WLSLLVPFV, the H-2 K^b binding peptide HBsAg$_{371-378}$ ILSPFLPL, the HLA-DR1 binding peptide HBsAg$_{180-195}$ QAGFFLLTRILTIPQS, the H-2 IA^b binding peptide HBsAg$_{126-138}$ RGLYFPAGGSSSG and the preS2 peptide HBsAg$_{109-134}$ MQWNSTTFHQTLQDPRVRGLYFPAGG were synthesized by Neosystem (Strasbourg, France) and dissolved in PBS-10 % DMSO at a concentration of 1 mg/ml. The numbering of the amino acid sequence of peptides starts from the first methionine of the HBV *ayw* subtype preS1 domain.

[0111] Immunization with DNA encoding the S2-S proteins of HBV

[0112] The pCMV-S2.S plasmid vector (Michel, M.L. et al., *Proc Natl Acad Sci U S A* **92**, 5307-5311 (1995)) coding for the preS2 and the S HBV surface antigens expressed under the control of the human CMV immediate early gene promotor was purified on Plasmid Giga Kit columns under endotoxin free conditions (Qiagen). Anesthesized mice were injected (50 µg each side) into regenerating tibialis anterior muscles, as previously described (Davis, H.L., Michel, M.L. & Whalen, R.G., *Hum Mol Genet* **2**, 1847-1851 (1993)).

T cell proliferation assay

[0113] Twelve days after the last immunization, red-blood cell-depleted, Ficoll-purified splenocytes (5.10^6 cells/ 25 cm^2 culture flask (Techno Plastic Products (TPP), Trasadingen, Switzerland)) were co-cultured with peptide-pulsed (20 µg/ml), γ-irradiated (180 Gy) LPS-blasts (5.10^6 cells/ culture flask) in RPMI medium supplemented with 10% FCS, 10 mM HEPES, 1 mM sodium pyruvate, 5 x 10^{-5} M 2-mercaptoethanol, 100 I.U/ml penicillin and 100 µg streptomycin, as described (Loirat, D., Lemonnier, F.A. & Michel, M.L., *J Immunol* **165**, 4748-4755 (2000)). On day 7, for proliferation assays, cells were plated (5 x 10^5 cells/well of flat bottomed 96 well microplates, (TPP)) with peptide-pulsed irradiated LPS-Blasts (2 x 10^5 cells/well) for 72 h in complete RPMI medium supplemented with 3% FCS. Cells were pulsed for the final 16 h with 1 µCi of (^{3}H)-thymidine per well before being harvested on filtermates with a TOMTEC collector (Perkin Elmer Applied Biosystem), and incorporated radioactivity was measured on a micro-β counter (Perkin Elmer Applied Biosystem). Results are given as stimulation index (SI) = cpm with specific peptide / cpm with irrelevant peptide.

[0114] Measurement of CTL activity

[0115] Cytotoxicity assays were performed on the same immune splenocyte populations as the proliferation assays. Responder cells (5.10^6 cells/ 25 cm² culture flask, TPP) and stimulating peptide-pulsed (20 µg/ml), γ-irradiated (180 Gy) LPS-blasts (5.10^6 cells/ culture flask) were co-cultured for 7 days in the same supplemented RPMI medium as for proliferation assays. Cytolytic activity was tested in a standard 4 h ^{51}Cr assay against RMA-S HHD target cells pulsed with 10 µg/ml of the experimental or control peptides. Specific lysis, in %, was calculated in duplicates, according to: [experimental -spontaneous release]/[maximal -spontaneous release] x 100, substracting the non-specific lysis observed with the control peptide.

[0116] Measurement of in vivo antibody production

[0117] At various times before and after DNA injection, blood was collected from mice by retrobulbar puncture with heparinized glass pipettes, and sera recovered by centrifugation were assayed for anti-HBs and anti-preS2 by specific ELISA. Purified recombinant particles containing HBV small S protein (1ug/ml) or preS2 (120-145) synthetic peptide (1ug/ml) were used as the solid phase. After blocking with PBST (PBS containing 0.1% Tween 20) supplemented with 10% FCS, serial dilutions were added. After extensive washing, the bound antibodies were detected with anti mouse Ig (total IgG) labeled with horseradish peroxidase (Amersham, Little Chalfont, UK). Antibody titers were determined by the serial end-point dilution method. Mouse sera were tested individually, and titers were the mean of at least three determinations. Serum dilutions below 1/100 were considered negative.

[0118] Antibody Titration

[0119] Sera from immunized mice were individually assayed by ELISA (Michel, M.L. et al., *Proc Natl Acad Sci U S A* 92, 5307-5311 (1995)) on either purified HBV middle and small proteins or preS2 synthetic $HBs_{109-134}$ peptide. After blocking with PBS 1x supplemented with 0.1% Tween 20, 10 % FCS and washings (x 3), bound antibodies were detected with horseradish peroxidase-labeled anti-mouse IgG (Amersham, Little Chalfont, UK). Antibody titers (means of at least 3 determinations) were determined by the serial end-point dilution method. Titers below 1/100 were considered negative.

[0120] <u>Vaccinia challenge and plaque assay</u>

[0121] DNA-injected mice were challenged intraperitoneally 12 days post last injection with 10^7 PFU of recombinant vaccinia virus (Western Reserve strain) expressing either the HbsAg (Smith, G.L., Mackett, M. & Moss, B., *Nature* 302, 490-495 (1983)) or the HBx protein (Schek, N., Bartenschlager, R., Kuhn, C. & Schaller, H., *Oncogene* 6, 1735-1744. (1991)) kindly provided, respectively, by Dr B. Moss and Dr H.Schaller. Four days later, ovaries were assayed for rVV titers by plaque assay on BHK 21 cells (Buller, R.M. & Wallace, G.D., *Lab Anim Sci* 35, 473-476 (1985).

Example 1: <u>Cell Surface Expression of MHC Molecules</u>

[0122] Cell surface expression of the HLA-A2.1, H-2 K^b/D^b, HLA-DR1, and H-2 IA^b molecules was evaluated on splenocytes by flow cytometry. As illustrated in Figure 1a, a similar level of HLA-A2.1 expression was observed in *HLA-A2.1-/HLA-DR1-transgenic, H-2 class I-/class II-KO* mice and *HLA-A2.1-transgenic, H-2 class I-KO* mice, while HLA-A2.1 was absent and H-2 K^b/D^b expressed exclusively in *HLA-DR1-*

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transgenic, *H-2 class II*-KO mice. Cell surface expression of HLA-DR1 and H-2 IA[b] was measured on B220[+]-enriched B cells. As shown in Figure 1b, a similar level of HLA-DR1 expression was observed in *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mice and *HLA-DR1*-transgenic, *H-2 class II*-KO mice, whereas no expression was detected in *HLA-A2.1*-transgenic, *H-2 class I*-KO mice. Cell surface expression of the transgenic molecules (especially HLA-DR1) was, however, lower than the expression of endogenous H-2 class I and class II molecules.

Example 2: Peripheral CD4^{+} and CD8^{+} T Cells

[0123] CD4^{+} and CD8^{+} splenic T cell numbers were determined by immunostaining and flow cytometry analysis as illustrated in Figure 2a.

[0124] CD4^{+} T cells represented 13-14% of the splenocyte population in both *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mice and *HLA-DR1*-transgenic, *H-2 class II*-KO mice. In contrast, only 2-3% of the cells were CD4^{+} in *H-2 class II*-KO mice (data not shown), in agreement with the initial report on mice lacking MHC class II molecules (Cosgrove, D. et al., *Cell* 66, 1051-1066 (1991)). As expected, expression of transgenic HLA-A2.1 molecules led to an increase in the size of the peripheral CD8^{+} T cell population, which reached 2-3% of the total splenocytes in both *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mice and *HLA-A2.1*-transgenic, *H-2 class I*-KO mice, compared to 0.6-1% in the $\beta2$ microglobulin ($\beta2m$)-KO MHC class I-deficient mice (Pascolo, S. et al., *J Exp Med* 185, 2043-2051 (1997)).

[0125] The results presented in Examples 1 and 2 show that:

(1)   In the HLA-A2$^+$HLA-DR1$^+$ β2m*IAβ^* mouse, the expression of HLA-A2 molecules, the absence of expression of H2-K^b molecules, the number of CD8$^+$ peripheral T-lymphocytes, and the diversity of the CD8$^+$ T repertoire are generally comparable to the HLA-A2$^+$ β2m* mouse;

(2)   In the HLA-A2$^+$HLA-DR1$^+$ β2m*IAβ^* mouse, the expression of HLA-DR1 molecules, the absence of expression of H2-IA^b molecules, the number of CD4$^+$ T-lymphocytes, and the diversity of the CD4$^+$ repertoire are generally comparable to the HLA-DR1$^+$IAβ^* mouse; and

(3)   The HLA-A2$^+$HLA-DR1$^+$ β2m*IAβ^* mouse has all the characteristic advantages found in HLA-A2$^+$ β2m* mice, and the HLA-DR1$^+$IA β^* mice.

Example 3: TCR BV Segment Usage

[0126] As the presence of a single MHC class I and single MHC class II molecule could diminish the size and diversity of the TCR repertoire, the expression of the various BV families and the CDR3 length diversity was studied as previously described (Cochet, M. et al., Eur J Immunol 22, 2639-2647 (1992)) by the RT-PCR-based immunoscope technique, on purified splenic CD4$^+$ or CD8$^+$ T cells. Peaks of significant magnitude with a Gaussian-like distribution were observed for most BV families (15 out of the 20 analyzed) in both CD8$^+$ (Figure 2b) and CD4$^+$ (Figure 2c) populations of T cells. Such profiles observed on peripheral T lymphocytes are typical of functionally rearranged BV segments with a 3 nucleotide length variation of the CDR3 subregions from one peak to the next (Cochet, M. et al., Eur J Immunol 22, 2639-2647 (1992)).

[0127] Absence of expansion (or profoundly altered profile) as observed for *BV*

5.3 and 17 were expected since these two *BV* segments are pseudogenes in C57BL/6

mice (Wade, T., Bill, J., Marrack, P.C., Palmer, E. & Kappler, J.W., *J Immunol* **141**,

2165-2167 (1988)); Chou, H.S. et al., *Proc Natl Acad Sci U S A* **84**, 1992-1996 (1987).

However, the altered profiles observed for *BV* 5.1, 5.2 and 11 segments were due to a

small subpopulation of corresponding *BV*-expressing T cells (they represent lower than

5% in C57BL/6 mice, and around 2% in *HLA-DR1*-transgenic *H-2 class II*-KO mice)

(data not shown). Other than these instances, both CD4$^+$ and CD8$^+$ T cells in *HLA-*

A2.1-/HLA-DR1-transgenic, *H-2 class I-/class II*-KO mice display, respectively, a pattern

of TCR *BV* chain usage and CDR3 diversity, which is similar to that of non-transgenic

C57BL/6 mice.

Example 4: Functional Characterization

[0128] HLA-A2$^+$HLA-DR1$^+$ β2m$^\cdot$IA$\beta^\cdot$ mice immunized with Ag HBs (hepatitis B

envelope protein) were analyzed. Figure 5 shows the specific humoral response, as

indicated by the production of HBs S2 antibodies. Figure 6 shows the specific DR1-

restricted CD4$^+$ T proliferation response of HBs$_{348-357}$. And Figure 7 shows the specific

HLA-A2-restricted CD8$^+$ cytolytic T response of the HBs$_{348-357}$ or HBs$_{335-343}$.

[0129] These results show that the HLA-A2$^+$HLA-DR1$^+$ β2m$^\cdot$IA$\beta^\cdot$ mouse allows

for simultaneous analysis of the specific humoral response, of the Ag-specific HLA-

DR1-restricted response of CD4$^+$ T helper cells, and of the cytolitic response of Ag-

specific HLA-A2-restricted CD8$^+$ T cells in an immunized individual.

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[0130] Additional data obtained from these mice is provided in the following

Tables 1-3.

Table 1. Proliferative responses of T CD4+ against HBV virus envelope HLA-DR1
epitopes from HLA-A2+DR1+H-2 CI-CII- transgenic mice injected with pcmv S2-S

position	Amino Acid sequence	Responder/tested mice	Stimulation Index
109-134	MQWNSTTFHQTLQDPRVRGLYFPAGG	(12/12)	3-4
200-214	TSLNFLGGTTVCLGQ	(6/12)	3-4
16/31	QAGFFLLTRILTIPQS	(12/12)	3-6
337/357	SLLVPFVQWFVGLSPTVWLSV	(5/12)	4-5

Table 2. Cytolytic response to HLA-A2+DR1+H-2 CI-CII-transgenic mice injected with
pcmv S2-S

position	Amino Acid sequence	Responder/tested mice	Maximal lysis
348-357	GLSPTVWLS	(12/12)	20-70%
335-343	WLSLLVPVF	(4/12)	30%

Table 3. Anti-PreS2 Antibody response anti of HLA-A2+DR1+H-2 CI-CII transgenic
mice injected with pcmv S2-S

position	Amino Acid sequence	Responder/tested mice
preS2	MQWNSTTFHQTLQDPRVRGLYFPAGG	(9/12)

Example 5: Immune Response to HBsAg-DNA-Vaccine

[0131] To evaluate the immunological potential of HLA-A2.1-/HLA-DR1-

transgenic, H-2 class I-/class II-KO mice, and to compare their humoral, CD4^{+} and CD8^{+}

T cell responses to those of humans, mice were immunized with an HBsAg-DNA plasmid. This plasmid encodes two hepatitis B virus envelope proteins (preS2/S middle and S/small) that self-assemble in particles carrying hepatitis B surface antigen. The currently used vaccine against hepatitis B comprises these two proteins.

[0132] As illustrated in Figure 3a for a representative mouse, HBsAg-specific antibodies were first detected at day 12 after injection of the HBsAg-DNA-vaccine (Figure 3a, upper panel), and the titer of these antibodies increased up to day 24 (12 days after the second DNA immunization, data non shown). This early antibody response was specific for the preS2-B cell epitope ($HBs_{109-134}$) carried by the middle HBV envelope protein and for HBsAg particles, in agreement with a similar response reported in HBsAg-DNA-immunized mice (Michel, M.L. et al., *Proc Natl Acad Sci U S A* 92, 5307-5311 (1995)) and in HBsAg vaccinated humans (Moulia-Pelat, J.P. et al., *Vaccine* 12, 499-502 (1994)).

[0133] The $CD8^+$ CTL response to HBsAg was examined to determine whether the $CD8^+$ T cells in the periphery of the *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mouse were functionally restricted by the transgenic human class I molecules. In HBV-infected $HLA-A2.1^+$ humans, the immunodominant HLA-A2.1-restricted HBsAg-specific CTL response is directed at the $HBsAg_{348-357}$ (Maini, M.K. et al., *Gastroenterology* 117, 1386-1396 (1999)) and at the $HBsAg_{335-343}$ (Nayersina, R. et al., *J Immunol* 150, 4659-4671 (1993)) peptide (i.e., a multi-epitopic response is observed). In C57BL/6 mice, the $H-2 K^b$-restricted HBsAg-specific CTL response is directed at the $HBsAg_{371-378}$ peptide (Schirmbeck, R., Wild, J. & Reimann, J., *Eur J Immunol* 28, 4149-4161 (1998)). To evaluate whether the humanized mouse may

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respond as humans, splenic T cells were restimulated for 7 days, as described herein, with either relevant (HBsAg$_{348-357}$, HLA-A2.1-restricted), or control (HBsAg$_{371-378}$, H-2 K^b-restricted; MAGE-3$_{271-279}$, HLA-A2.1-restricted) peptide. Figure 3a (middle panel) shows that HBsAg-DNA-immunization elicited a strong HBsAg$_{348-357}$-specific CTL response, but no response to either HBsAg$_{371-378}$ or the MAGE-3$_{271-279}$ peptide.

[0134] To determine whether the CD4$^+$ T cells in the periphery of this *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class II*-KO mouse may be functionally restricted by the transgenic human class II molecules, the CD4$^+$ T cell response to the HBsAg protein was examined. In HBsAg-vaccinated or HBV-infected HLA-DR1$^+$ humans, an immunodominant HLA-DR1-restricted HBsAg-specific CD4$^+$ T cell response is directed at the HBsAg$_{180-195}$ peptide (Mm, W.P. et al., *Hum Immunol* 46, 93-99 (1996)). In C57BL/6 mice, the H-2 IA^b-restricted HBsAg-specific CD4$^+$ T cell response is directed at the HBsAg$_{126-138}$ peptide (Milich, D.R., *Semin Liver Dis* 11, 93-112(1991)). To compare the humanized mouse with humans and wild-type mice, splenic T cells were restimulated *in vitro* with either relevant (HBsAg$_{180-195}$, HLA-DR1-restricted) or control (HBsAg$_{126-138}$, H-2 IA^b-restricted; HIV 1 Gag$_{263-278}$, HLA-DR1-restricted) peptides. Figure 3a (lower panel) shows a strong proliferative response directed against the HLA-DR1-restricted HBsAg$_{180-195}$ peptide, while the H-2 IA^b-restricted peptide was not efficient at stimulating a response, as expected. Similarly, no response was induced by the HIV 1 Gag$_{263-278}$ peptide. Moreover, an additional *in vitro* recall with the HBsAg$_{180-195}$ peptide increased several-fold the specific proliferative index (data not shown).

[0135] Having documented in a first HBsAg-DNA-immunized *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class I-/class II*-KO mouse the development and the specificity of

the HBsAg-specific antibody, proliferative and cytolytic T cell responses, 6 additional

HBsAg-DNA-immunized and 6 naive control *HLA-A2.1-/HLA-DR1*-transgenic *H-2 class*

I-/class II-KO mice were also tested individually for the same three responses. As

illustrated in Figure 3b, the three reponses were simultaneously documented in the 6

immunized animals tested and not in control naive mice. Interestingly, 2 immunized

mice were able to develop CTL responses against both HBsAg$_{348-357}$ and HBsAg$_{335-343}$

HLA-A2.1 restricted peptides (Figure 3b, middle panel).

Example 6: **Protection Assays**

[0136] The above examples document the induction of HBsAg-specific humoral,

CD4$^+$ and CD8$^+$ T cell responses in *HLA-A2.1-/HLA-DR1*-transgenic, *H-2 class I-/class*

II-KO mice, and show that they are directed at the same immunodominant epitopes as

those of naturally-infected or HBsAg-vaccinated humans. This example tested whether

these responses conferred protection to vaccinated animals. Since mice are not

permissive to HBV, a HBsAg-recombinant vaccinia virus (rVV-HBsAg) was used for

these experiments. Mice were immunized twice intramuscularly with 100 μg of HBsAg-

DNA. Twelve days after the last immunization, mice were challenged intraperitoneally

with 10^7 PFU of rVV-HBsAg. Four days later, virus titers were determined according to

published methods and recorded as rVV PFU/ovary (Buller, R.M. & Wallace, G.D., *Lab*

Anim Sci 35, 473-476 (1985)).

[0137] The results are illustrated in Figure 4. Naive animals that had not been

immunized with HBsAg-DNA showed evidence of rVV-HBsAg replication after

challenge. In contrast, the virus titers in mice immunized with HBsAg-DNA were more

than 4 orders of magnitude lower. These results strongly suggest that vaccination with HBsAg-DNA induced protective HBsAg-specific immune responses that controlled the infection with rVV-HBsAg.

[0138] The specificity of the protection conferred by HBsAg-DNA-vaccination was documented by challenging HBsAg-DNA-immunized mice with another HBx-recombinant VV (encoding hepatitis B x protein). No reduction of rVV-HBx replication was observed in HBsAg-DNA-immunized mice compared to unimmunized controls.

Example 7: HLA-DR1-Restricted CD4$^+$ T Cells Are Critical for Antibody and CTL Responses and Protection Against Viral Infection

[0139] To evaluate whether HLA-DR1-restricted T helper lymphocytes contribute to antibody and CTL responses in the humanized mice, the immune response and the efficiency of viral infection were compared in single (*HLA-A2.1*) and double (*HLA-A2.1/HLA-DR1*) transgenic, *H-2 class I-/class II*-KO mice. As shown in Table 4, a potent HBsAg$_{348-357}$-specific CTL response was observed in *HLA-A2.1-/HLA-DR1*-double transgenic, *H-2 class I-/class II*-KO mice, but not in *HLA-A2.1*-single transgenic *H-2 class I-/ class II*-KO mice. Furthermore, anti-HBs antibodies could not be detected in HBsAg-DNA-vaccinated *HLA-A2.1*- single transgenic *H-2 class I-/ class II*-KO mice. As a consequence, HBsAg-DNA-immunized *HLA-A2.1*- single transgenic *H-2 class I-/ class II*-KO mice were not protected against rVV-HBsAg infection.

TABLE 4

Table 4 Antibody, cytolytic, and proliferative responses of HBsAg- DNA-immunized mice, and protection against rVV-HBsAg-challenge

	Mice	Specific Lysis (%)		Proliferation (SI)	Antibody	rVV-HBsAg
		348-357	335-343	179-194	Titer	PFU/ovary (log10)
A	1	0	0	1	0	$2.5.10^8$
	2	0	0	1	0	$2.5.10^8$
	3	0	0	1	0	10^8
	4	0	0	1	0	$2.5.10^8$
	5	0	0	1	0	10^8
	6	0	0	1	0	$1.5.10^8$
B	1	30	15	4.7	2000	10^4
	2	14	0	3.9	3000	3.10^3
	3	30	11	4	7500	4.10^3
	4	5	0	2.5	6500	$7.5.10^3$
	5	50	30	6.3	13000	$7.5.10^2$
	6	40	18	4	16000	5.10^2
	7	6	7	2.9	1500	2.10^4
	8	5	5	3	2500	$1.5.10^4$
	9	24	36	4.5	3000	$<10^2$
	10	23	14	5	15000	5.10^3
C	1	0	0	1	0	10^8
	2	0	0	1	0	2.10^8
	3	0	0	1	0	$1.5.10^8$
	4	0	0	1	0	10^8
	5	0	0	1	0	$2.5.10^8$
	6	0	0	1	0	10^8

Naive *HLA-A2.1-/HLA-DR1*-double transgenic *H-2 class I-/class II*-KO mice (A 1-6), HBsAg-DNA-immunized *HLA-A2.1-/HLA-DR1*-double transgenic *H-2 class I-/class II*-KO mice (B 1-10) and HBsAg-DNA-immunized *HLA-A2.1-* single transgenic *H-2 class I-/ class II*-KO mice (C 1-6) were challenged intraperitoneally with 10^7 PFU of rVV-HBsAg. Four days later, PFU per ovary, cytolytic and proliferative splenic T cell responses and serum antibody titers were assessed individually using either $HBsAg_{348-357}$, (immunodominant) or $HBsAg_{335-343}$ (subdominant), HLA-A2.1-restricted peptides-loaded RMAS-HHD target cells (E/T ratio 30/1) for cytolytic assays, $HBsAg_{179-194}$ HLA-DR1-restricted peptide for

proliferation assays and preS2$_{109-134}$ peptide for the determination of antibody (IgG) titers.

[0140] The entire contents of all references, patents and published patent applications cited throughout this application are herein incorporated by reference in their entirety.